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ATTACHMENT A

Expression cloning of a human corticotropin-releasing-factor receptor

(G protein-coupled receptor/hypothalamic–pituitary–adrenocortical axis/cyclic AMP/peptide hormone receptor)

RUOPING CHEN, KATHY A. LEWIS, MARILYN H. PERRIN*, AND WYLIE W. VALE

The Clayton Foundation Laboratories for Peptide Biology, The Salk Institute, La Jolla, CA 92037

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ABSTRACT **Corticotropin-releasing factor (CRF) is the principal neuroregulator of the hypothalamic–pituitary–adrenocortical axis and plays an important role in coordinating the endocrine, autonomic, and behavioral responses to stress and immune challenge. We report here the cloning of a cDNA coding for a CRF receptor from a human corticotropic tumor library. The cloned cDNA encodes a 415-amino acid protein comprising seven putative membrane-spanning domains and is structurally related to the calcitonin/vasoactive intestinal peptide/growth hormone-releasing hormone subfamily of G protein-coupled receptors. The receptor expressed in COS cells binds rat/human CRF with high affinity (K_d = 3.3 ± 0.45 nM) and specificity and is functionally coupled to adenylate cyclase. The CRF antagonist α-helCRF-(9–41) inhibits the CRF-stimulated increase in intracellular cAMP. Northern blot analysis reveals that the CRF receptor is expressed in the rat pituitary and brain as well as in the mouse AtT20 corticotropic cells. We also describe an alternatively spliced form of the receptor which includes an insert of 29 amino acids in the first intracellular loop.**

Corticotropin-releasing factor (CRF) (1) is a 41-amino acid peptide isolated from the hypothalamus by virtue of its ability to stimulate the production of adrenocorticotropic hormone (ACTH) and other proopiomelanocortin products of the anterior pituitary. Anatomic, functional, and pharmacologic studies have suggested numerous physiologic roles for CRF within the brain, adrenals, gonads, gastrointestinal tract, placenta, and sites of inflammation (2, 3). A subset of CRF neurons may coordinate many of the endocrine, autonomic, and behavioral responses to stress and may be involved in the pathophysiology of affective disorders. Moreover, CRF appears to be a key intermediary in the communication between the immune systems and the central nervous and endocrine systems (4–6). The neuropeptide and its binding sites are broadly distributed throughout the central nervous system as well as in some peripheral sites, reflecting the role CRF may play in endocrine, autonomic, and behavioral aspects of the stress response, as well as in such pathophysiologic states as depression, anxiety, and anorexia nervosa.

CRF exerts its effects by initially binding to a membrane-bound receptor. High-affinity CRF binding sites have been characterized in the rat (7) and human (8) pituitary, rat (9) and human (10) brain, and on AtT20 cells (11), a mouse corticotropic tumor cell line. The CRF receptor is coupled to adenylate cyclase (12–15), probably through a GTP-binding protein (16). Although a high-affinity soluble binding protein for CRF was recently isolated from human plasma (17) and cloned (18), the membrane receptor for this important neuropeptide was not identified.

Here we present the expression cloning and functional expression of a cDNA encoding a CRF receptor from a human Cushing corticotropic cell tumor. We also report a variant of the receptor produced by alternative splicing.†

MATERIALS AND METHODS

Library Construction. A human corticotropic adenoma removed from a patient with Cushing disease was generously provided by Mary Lee Vance and the neurosurgery unit headed by Edward Laws (University of Virginia School of Medicine). Total RNA was prepared by guanidine extraction and poly(A)$^+$ RNA was obtained with oligotex-dt (Qiagen). The corresponding cDNA was ligated into pcDNA1 (Invitrogen), yielding a library of ≈1.5 × 10^6 primary recombinants, 80% of which contained inserts. A λZAP II (Stratagene) library was synthesized from the same human Cushing tumor cDNA by using *Not* I/*Eco*RI adapters.

Expression Cloning. Expression screening of the pcDNA1 library was carried out as reported (19). Binding to transfected COS-M6 cells was assessed by incubation with 1 × 10^6 cpm of [^{125}I]iodotyrosyl ovine CRF (Peninsula Laboratories) [iodinated as described (16)] in 0.7 ml of binding buffer [0.1% ovalbumin in HDB (25 mM Hepes/137 mM NaCl/5 mM KCl/0.7 mM Na_2HPO_4, pH 7.5)] for 90 min at 21°C.

Phage Library Screening. A 1.2-kb *Pst* I fragment in the coding region of hct-0 was used to screen the λZAP II library by standard methods. Double-stranded sequencing was performed by the dideoxy chain-termination method with the Sequenase kit (United States Biochemical).

Radioreceptor Assay of Cloned Receptor. Two days after transfection with 10–20 μg of plasmid DNA, the COS-M6 cells were washed with HDB and detached by incubation with 0.5 mM EDTA in HDB for 15 min at 21°C. The cells were washed twice with HDB and homogenized in 5% sucrose. The homogenate was centrifuged at 600 × *g* for 5 min, after which the supernatant was centrifuged at 40,000 × *g* for 20 min. The resulting membrane homogenate, P_2, was resuspended at 1–4 mg/ml in 10% sucrose and used in the binding assay as described (16). Dissociation constants were calculated from relative potencies by using the ALLFIT program (20) and determined from competitive displacement assays with rat/human CRF (r/hCRF) as the standard.

cAMP Accumulation Assay. COS-M6 cells were transfected as described (21). At least 2 hr before treatments, the medium was changed to contain 0.1% serum. After a 30-min preincubation in medium with or without 0.1 mM 3-isobutyl-1-

Abbreviations: ACTH, adrenocorticotropic hormone; CRF, corticotropin-releasing factor; r/hCRF, rat/human CRF; GnRH, gonadotropin-releasing hormone; GRF, growth hormone-releasing factor; IBMX, 3-isobutyl-1-methylxanthine; VIP, vasoactive intestinal peptide.

*To whom correspondence should be addressed.

†The sequences reported in this paper have been deposited in the GenBank database (accession nos. L23332 and L23333).

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methylxanthine (IBMX), test substances were added and incubated for 30 min at 37°C. The cells were extracted as described (12). cAMP levels were determined in duplicate from triplicate wells with an RIA kit (Biomedical Technologies, Stoughton, MA).

Northern Blot Analysis. Poly(A)$^+$ RNA was size-fractionated in a denaturing formaldehyde/agarose gel and transferred to nitrocellulose paper. The blot was prehybridized for 15 min at 68°C in the QuikHyb hybridization solution (Stratagene) plus salmon sperm DNA (100 μg/ml) and hybridized in the same solution at 68°C for 30 min to a 1.3-kb *Pst* I cDNA fragment labeled by random priming (Amersham). The *Pst* I fragment includes the majority of the coding region of CRF-R_1. The blot was washed twice at 21°C in 2× SSPE/0.1% SDS for 15 min and once at 60°C in 0.2× SSPE/0.1% SDS for 30 min (1× SSPE is 0.18 M NaCl/10 mM sodium phosphate, pH 7.4/1 mM EDTA).

RESULTS

Cloning of CRF-R_1. From a screen of ≈400,000 independent clones, 1 positive pool was found. A subdivision of this pool yielded a single clone, hct-0, that expressed high-affinity binding when transfected into COS-M6 cells. The sequence of this and of subsequent clones revealed the inclusion of two putative intronic sequences separated by an exon. To obtain other clones that did not include the introns, a phage library created from the same tumor was screened at high stringency with a probe generated from the original clone. Three positives were identified, two of which, CRF-R_1 and CRF-R_2, were sequenced and found to lack introns.

Structural Characteristics of the CRF Receptor. Hydropathy analysis (22) of the CRF-R_1 amino acid sequence, shown in Fig. 1, indicates eight hydrophobic regions of ≈20 amino acids corresponding to a possible signal peptide at the N terminus and seven putative transmembrane domains. CRF-R_1 contains a 2584-bp insert with a 1245-bp open reading frame encoding a 415-amino acid protein. Sequence analysis (Fig. 2) shows that the CRF receptor appears to belong to the calcitonin/vasoactive intestinal peptide (VIP)/growth hormone-releasing factor (GRF) family of G protein-coupled receptors (23–29). At the amino acid level, the percent identity to the human GRF receptor is 31%, to the rat secretin receptor, 32%, to the rat VIP receptor, 30%, and to the porcine calcitonin receptor, 25%. However, there is significantly greater homology throughout the transmembrane domains, so that the CRF receptor is 44%, 46%, 43%, and 38% identical with the GRF, secretin, VIP, and calcitonin receptors, respectively, in those regions.

Five potential N-glycosylation sites (Asn-Xaa-Ser/Thr) in the N terminus are shown in Fig. 2. Also shown are potential protein kinase C phosphorylation sites in the first and second intracellular loops and in the C-terminal tail, as well as a casein kinase II and protein kinase A phosphorylation site in the third intracellular loop (30). The third clone, CRF-R_2, is an alternatively spliced form of the receptor in which 29 amino acids are inserted into the first intracellular loop. No new putative phosphorylation sites are introduced in the alternatively spliced form of the receptor.

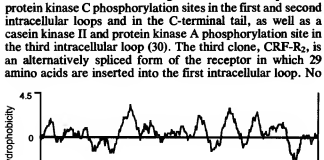

FIG. 1. Kyte–Doolittle (22) hydrophobicity analysis of CRF-R_1.

Binding Characteristics of the Cloned Receptor. The cloned CRF receptor hct-0 exhibited appropriate pharmacologic specificity, with high affinity for r/hCRF (Fig. 3) [K_d = 3.3 ± 0.45 nM (n = 4)], ovine CRF [K_d = 8.3 (4–16) nM (n = 1)], and for the antagonist (2) α-helCRF-(9–41) [K_d = 1.0 ± 0.10 nM (n = 2)] and low affinity for the biologically impotent analog [Ala^{14}]ovine CRF [K_d > 300 nM (n = 2)]. hGRF-(1–40)-OH, salmon calcitonin, and VIP were inactive in the receptor assay. Cells transfected with the rat gonadotropin-releasing hormone (GnRH) receptor (21) showed no binding of CRF. The CRF-R_1 clone displayed similar affinity to that of hct-0 [K_d = 3.8 ± 0.20 nM (n = 1)] for r/hCRF.

Intracellular cAMP Accumulation in Cells Transfected with the Cloned Receptor. Consistent with earlier observations implicating adenylate cyclase in the transduction mechanism of the native CRF receptor (12–15), COS-M6 cells transfected with the hct-0 clone responded to r/hCRF with 10- to 20-fold increases in intracellular cAMP, whereas COS-M6 cells transfected with the rat GnRH receptor did not respond at all. The response was dose-dependent (Fig. 4*A*), and the half-maximal dose of ≈3 nM CRF corresponded to the K_d obtained from the radioreceptor assay and to the EC_{50} determined for CRF-stimulated cAMP production by rat anterior pituitary cells (12). hGRF-(1–40)-OH, salmon calcitonin, and VIP had no effect on cAMP levels in the cells transfected with the cloned receptor (Fig. 4*B*). The CRF antagonist α-helCRF-(9–41) blocked the induction of cAMP by r/hCRF (Fig. 5). A comparison of the two clones hct-0 and CRF-R_1 showed the same dose–response of r/hCRF-stimulated intracellular cAMP accumulation and the same lack of response to GRF, calcitonin, and VIP.

Tissue Distribution of the Cloned Receptor. Autoradiographic and biochemical techniques have detected the native CRF receptor in the pituitary (7) and various brain regions (9). Northern blot analysis of poly(A)$^+$ RNA using the CRF-R_1 probe revealed the presence of a 2.7-kb mRNA in rat brain and pituitary and in mouse AtT20 corticotropic cells (Fig. 6).

DISCUSSION

Using an expression cloning approach we have isolated a CRF receptor cDNA from a human Cushing tumor. When the binding assay was used to monitor receptor expression it was largely masked by high nonspecific binding of the CRF radioligand. Substitution of ovalbumin for bovine serum albumin was found to reduce the nonspecific binding of ^{125}I-labeled ovine CRF, allowing positive cells to be detected.

Based on a hydropathy analysis, the receptor has a hydrophobic domain at the N terminus that may encode a signal peptide. There are seven other hydrophobic regions characteristic of membrane-spanning domains. After removal of the putative signal peptide, the receptor is predicted to have a molecular weight of ≈44,000.

There are six conserved cysteines in the calcitonin/VIP/GRF family of receptors. These cysteines may be involved in the folding of the extracellular domain allowing binding of the ligands. The conservation of all these cysteines in the CRF receptor may serve the same purpose or may merely reflect common ancestry.

Although r/hCRF binds with high affinity to the soluble CRF-binding protein (CRF-BP) (17, 18) it has no sequence homology with the cloned receptor. Because the affinity of ovine CRF is nearly 100 times lower for the human CRF-BP than for the cloned receptor, there must be different structural determinants for binding CRF ligands. This difference in specificity was exploited in the expression cloning by using an ovine analog of CRF as the radioligand to avoid detecting

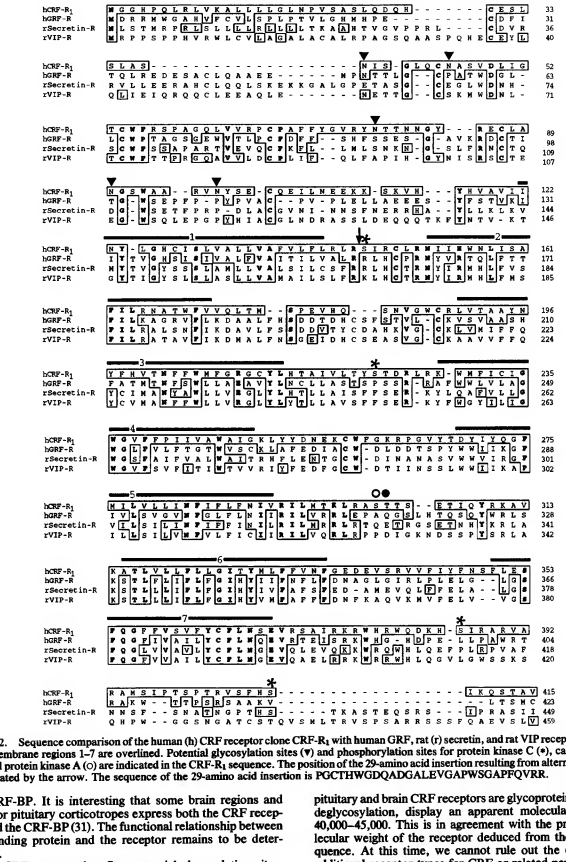

FIG. 2. Sequence comparison of the human (h) CRF receptor clone CRF-R$_1$ with human GRF, rat (r) secretin, and rat VIP receptors. Putative transmembrane regions 1–7 are overlined. Potential glycosylation sites (▼) and phosphorylation sites for protein kinase C (•), casein kinase II (■), and protein kinase A (○) are indicated in the CRF-R$_1$ sequence. The position of the 29-amino acid insertion resulting from alternative splicing is indicated by the arrow. The sequence of the 29-amino acid insertion is PGCTHWGDQADGALEVGAPWSGAPFQVRR.

the CRF-BP. It is interesting that some brain regions and anterior pituitary corticotropes express both the CRF receptor and the CRF-BP (31). The functional relationship between the binding protein and the receptor remains to be determined.

The CRF receptor has five potential glycosylation sites. Their presence may explain the fact that the apparent molecular size of the pituitary receptor seen in crosslinking experiments (32) is larger than that predicted from the size of the cloned receptor. Further, it has been shown (33) that both the pituitary and brain CRF receptors are glycoproteins that, after deglycosylation, display an apparent molecular weight of 40,000–45,000. This is in agreement with the predicted molecular weight of the receptor deduced from the cDNA sequence. At this time, we cannot rule out the existence of additional receptor types for CRF or related peptides in the brain or elsewhere.

There are potential phosphorylation sites in each of the three intracellular loops, as well as in the C terminus. The protein kinase C phosphorylation sites may be involved in

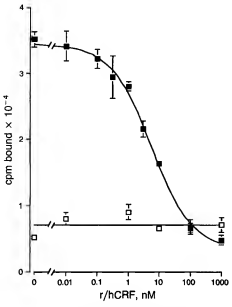

FIG. 3. Competitive displacement by r/hCRF of ^{125}I-labeled [Nle^{21},Tyr^{32}]ovine CRF bound to membranes prepared from COS-M6 cells transfected with hct-0 (■) or rat GnRH receptor (□). Data are from one representative experiment repeated at least four times.

modulating the signal transduction as evidenced by the potentiation of CRF-stimulated cAMP accumulation and ACTH release (34, 35) by activators of protein kinase C, such as phorbol esters and vasopressin. Protein kinase A phosphorylation of residues in the third intracellular loop and the C terminus has been implicated in the desensitization and down-regulation of other G protein-coupled receptors (36). Whether the phosphorylation sites in the CRF receptor are involved in the observed CRF desensitization (37) remains to be investigated. Consistent with the coupling of the CRF receptor to the adenylate cyclase system, the third intracellular loop of CRF-R$_1$ contains a sequence similar to the G_s-activating region found in the third intracellular loop of the β_2-adrenergic receptor (38).

Our original clone contains an in-frame insertion at a point identified as a consensus sequence for an intron–exon splice junction; consequently, the CRF receptor gene contains at least two introns. The gene for the GRF receptor contains many introns within the coding region (23). Alternative splicing events produce an insertion of 41 amino acids in the third intracellular loop of the rat GRF receptor (23, 25), and a deletion in the first extracellular loop of the mouse GRF receptor (25). In the only alternative form of the CRF receptor identified thus far, 29 amino acids are inserted in the first intracellular loop. It is possible that alternative RNA processing may generate two different forms of the CRF receptor. The functional significance of these forms remains to be elucidated.

Because the CRF receptor was cloned from a human pituitary tumor, the receptor may include mutations compared to the normal receptor. However, the affinity and specificity of the cloned receptor are similar to those of the normal rat pituitary receptors, as are the transduction of CRF-stimulated accumulation of intracellular cAMP and the antagonism by the potent CRF antagonist α-helCRF-(9–41).

When expression of the cloned CRF receptor was examined by Northern analysis (Fig. 6), a major transcript of 2.7 kb was detected in the mouse corticotropic cell line AtT20 and in rat brain and pituitary, but not in rat heart. These results are consistent with the previous studies of the pituitary and brain as the primary target sites of CRF action and the identification of CRF receptors in these tissues.

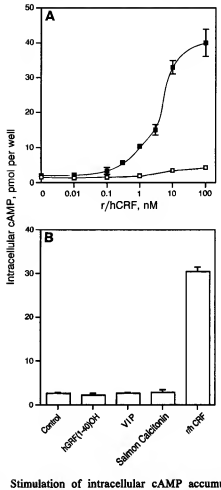

FIG. 4. Stimulation of intracellular cAMP accumulation in COS-M6 cells transfected with hct-0. Data are from one representative experiment repeated at least three times. (*A*) Stimulation by r/hCRF of cAMP accumulation in cells pretreated (■) or not treated (□) with IBMX. (*B*) cAMP accumulation after treatment with r/hCRF (100 nM) or non-CRF peptides (100 nM) in cells pretreated with IBMX.

In conclusion, we have cloned from a human pituitary tumor a cDNA encoding a 415-amino acid protein that displays the functional properties of a physiological CRF re-

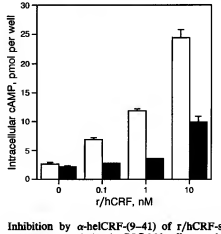

FIG. 5. Inhibition by α-helCRF-(9–41) of r/hCRF-stimulated intracellular cAMP accumulation in COS-M6 cells transfected with hct-0 and pretreated with IBMX. r/hCRF was added without (open bars) or with (filled bars) 2 μM α-helCRF-(9–41).

FIG. 6. Expression of CRF receptor as shown by Northern blot analysis of poly(A)$^+$ RNA (4 μg) from AtT20 cells and rat brain, pituitary, and heart. The autoradiograph shown is a 5-hr exposure. Positions of 28S and 18S rRNA and size of the mature CRF-R mRNA are indicated.

ceptor—namely, high-affinity CRF binding and transduction of the accumulation of cAMP in response to CRF. The sequence confirms that the receptor belongs to a G protein-coupled receptor family characterized by seven transmembrane domains. The cloning of the human CRF receptor fills a major gap in our knowledge of the signaling molecules of the hypothalamic–pituitary–adrenocortical axis and provides tools to further explore the mode of action of CRF and its target cells.

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